TonEBP is inhibited by RNA helicase A via interaction involving the E'F loop

Emanuela COLLA, Sang D. LEE¹, Mee R. SHEEN, Seung K. WOO and H. Moo KWON²

Department of Medicine, University of Maryland, 22 South Greene Street, Suite N3W143, Baltimore, MD 21201, U.S.A.

TonEBP [TonE (tonicity-responsive enhancer)-binding protein] is a transcriptional activator of the Rel family like NF- κ B (nuclear factor κ B) and NFAT (nuclear factor of activated T-cells). TonEBP plays a key role in the protection of cells in the kidney medulla from the deleterious effects of hyperosmolality. This is achieved by enhancing expression of HSP70 (heat-shock protein 70) and other genes whose products drive cellular accumulation of organic osmolytes. TonEBP is stimulated by ambient hypertonicity via multiple pathways that regulate nuclear translocation and transactivation. In the present paper, we report that TonEBP is associated *in vivo* with RHA (RNA helicase A). The N- and C-termini of RHA bound the E'F loop of the DNA-binding domain of TonEBP. The interaction was not affected by DNA binding or dimerization of TonEBP. Overexpression of RHA inhibited the

activity of TonEBP; however, catalytic activity of RHA was dispensable for the inhibition. When the ambient tonicity was raised, the TonEBP–RHA interaction decreased, suggesting that dissociation of RHA is a pathway to stimulate TonEBP. We conclude that the E'F loop of TonEBP interacts with RHA like NFAT and NF- κ B interact with AP1 (activator protein 1) and the high-mobility group protein HMG-I(Y) respectively. While RHA interacts with and stimulates other transcription factors such as CREB (cAMP-response-element-binding protein), NF- κ B and mineralocorticoid receptor, it inhibits TonEBP.

Key words: E'F loop, hypertonicity, nuclear factor κ B (NF- κ B), RNA helicase A (RHA), small interfering RNA (siRNA), tonicity-responsive-enhancer-binding protein (TonEBP).

INTRODUCTION

TonEBP [TonE (tonicity-responsive enhancer)-binding protein] was cloned as a *trans*-acting factor for the *cis*-element TonE that mediated stimulation of transcription by hypertonicity [1]. TonEBP is also called NFAT5 (nuclear factor of activated T-cells 5) as it was cloned independently, based on its similarity to NFAT transcription factors [2]. TonEBP belongs to the Rel family of DNA-binding proteins that are defined by the structural similarity in the DNA-binding region, the so-called Rel-homology domain [3]. NFAT and NF- κ B (nuclear factor κ B) are other members of the Rel family.

When ambient tonicity or the concentration of salt is raised, TonEBP is stimulated via multiple pathways including nuclear translocation [4], increased transactivation (stimulation of transcription) [5] and induction [6]. TonEBP plays a major role in the adaptation to hyperosmolality, especially in the kidney medulla where osmolality is very high owing to high concentrations of salt and urea. TonEBP stimulates the transcription of membrane transporters and aldose reductases that catalyse the cellular accumulation of organic osmolytes [7], which results in the protection of cells from the deleterious effects of hypertonicity [8]. TonEBP also stimulates expression of HSP70 (heat-shock protein 70) that protects cells in the renal medulla from high concentrations of urea [9]. Embryonic fibroblasts established from TonEBP^{−/−} mice do not survive in hypertonic conditions while embryonic fibroblasts from TonEBP^{+/+} mice do [10]. More than 95 % of TonEBP^{−/−} foetal mice die in late gestational stages [10,11]. Surviving

TonEBP^{−/−} mice display severe atrophy of the kidney medulla because of cell death from failure to adapt to the hypertonicity [11].

Adaptation to hypertonicity also appears to be a major function of TonEBP in non-renal tissues whose osmolality is normally near isotonic and far below the level seen in the kidney medulla. In the thymus and spleen, osmolality is approx. 30 mosmol/kg higher than other organs and serum [10]. While T-cells from TonEBP^{+/+} mice tolerate this mild hyperosmolality, those from TonEBP^{+/−} mice display a markedly reduced ability to proliferate [10]. In TonEBP^{+/−} mice [10] and transgenic mice expressing a dominant-negative form of TonEBP in the thymus [12], the number of T-cells is significantly reduced owing to the sensitivity of the T-cells to the hyperosmolarity in lymphoid tissues. These data illustrate the importance of TonEBP in the adaptation of T-cells to hypertonicity. In brain, TonEBP is expressed primarily in neurons where it is localized in the nucleus [13]. TonEBP in the neuron increases dramatically in response to systemic hypernatraemia, suggesting that TonEBP plays a role in the adaptation to hypertonicity [13].

The molecular mechanism underlying the hypertonicity-induced stimulation of TonEBP is of great interest. In TonEBP, there are multiple independent domains that are involved in transactivation [5]. Only a subset of the transactivation domains is stimulated by hypertonicity. In the present study, we report that TonEBP binds specifically to RHA (RNA helicase A). The binding inhibits the activity of TonEBP. Hypertonicity reduces the binding, leading to stimulation of TonEBP.

Abbreviations used: AP1, activator protein 1; CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; GST, glutathione S-transferase; HEK-293T, human embryonic kidney expressing the simian virus 40 T antigen; HMG, high-mobility group; Hsc70, heat-shock cognate 70; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MEF1, murine elongation factor-1; mIMCD3, renal inner medullary collecting duct cell line established from a transgenic mouse expressing the simian virus 40 T-antigen; NFAT, nuclear factor of activated T-cells; NF- κ B, nuclear factor κ B; RHA, RNA helicase A; siRNA, small interfering RNA; SV40, simian virus 40; TonE, tonicity-responsive enhancer; TonEBP, TonE-binding protein; T-TBS, Tris-buffered saline with Tween 20.

¹ Present address: Department of Physiology, Chungnam National University, Daejeon, South Korea.

² To whom correspondence should be addressed (email mkwon@medicine.umaryland.edu).

EXPERIMENTAL

Cell culture, cell lysates, and nuclear and cytoplasmic extracts

Jurkat cells (human leukaemia T-cell line) were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS (foetal bovine serum) (Gemini Bio-Products), 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies). COS7, HeLa, and HEK-293T [human embryonic kidney expressing the SV40 (simian virus 40) T-antigen] cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS and antibiotics as above. mIMCD3 (renal inner medullary collecting duct cell line established from a transgenic mouse expressing the SV40 T-antigen [14]) cells were cultured in an equal mixture of DMEM and F12 supplemented with 10% (v/v) FBS and antibiotics. Where indicated, cells were switched to hypertonic medium made by the addition of 100 mM NaCl to regular medium. For pull-down assays using GST (glutathione S-transferase)-Yc1, Jurkat cell lysate was prepared as follows. Cells were washed by centrifugation at 5000 g for 10 min in PBS and resuspended in ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT (dithiothreitol) and protease inhibitor cocktail (Roche Molecular Biochemicals). After two rounds of sonication each for 15 s, the lysate was incubated for 30 min at 4°C with gentle mixing and cleared by a centrifugation at 12000 g for 30 min. Where indicated (see Figure 6), COS7 cells were fractionated into nuclear and cytoplasmic extracts using NE-PER® Nuclear and Cytoplasmic Extraction kit (Pierce).

DNA constructs

The N-terminal TonEBP fragment Yc1 (see Figure 2A) was fused to GST by cloning in pGEX (Amersham Biosciences) for bacterial expression. Fragments of RHA in pGEX [15] (see Figure 3) were gifts from Dr Jeffrey Parvin (Harvard Medical School, Boston, MA, U.S.A.). Full-length wild-type RHA and K417E mutant [16] were gifts from Dr Chee-Gun Lee (New Jersey Medical School, Newark, NJ, U.S.A.). TonEBP and its fragments were described previously [17]. For expression in cultured mammalian cells, appropriate cDNA was fused to FLAG or Myc using pCMV-Tag3 or pCMV-Tag2 (Stratagene). Truncated and deleted TonEBP fragments were made using standard techniques and were verified by DNA sequencing. The TonE-driven luciferase reporter in pGL2 promoter (Promega) was described previously [18].

Pull-down assay using GST-fusion

For production of GST-fusion protein, bacteria harbouring pGEX with a cDNA insert (see above) were grown to mid-exponential phase and treated with 0.2 mM IPTG (isopropyl β-D-thiogalactoside) (Sigma-Aldrich) for 7 h at 30°C to induce expression. The bacterial cells were resuspended in lysis buffer, sonicated and incubated for 30 min at 4°C as described above. After clearing by centrifugation at 12000 g for 15 min, the lysate was incubated with GSH conjugated to agarose for 1 h with gentle mixing. GST-fusion protein bound to the GSH-agarose was washed extensively with lysis buffer by centrifugation at 10000 g for 10 min. To screen proteins interacting with the N-terminus of TonEBP, 1 mg of purified GST-Yc1 or GST bound to GSH-agarose was incubated with 6 mg of protein of Jurkat cell lysate for 1 h at 4°C with gentle mixing. The GST-Yc1-agarose or GST-agarose was extensively washed by centrifugation at 10000 g for 10 min and resuspended in SDS sample buffer. The solution was heated for 5 min at 95°C and electrophoresed on an SDS/4–15% gradient polyacrylamide gel (Bio-Rad). The gel was stained with Coomassie Blue. Bands unique to GST-Yc1 were cut

and analysed by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight)-MS. To confirm the protein interaction, the GST pull-down was performed on a smaller scale, separated on an SDS/polyacrylamide gel, and transferred on to a nitrocellulose membrane (Bio-Rad). The membrane was stained with Ponceau S to view the amount of fusion proteins and then immunoblotted as described below. To map the sites of TonEBP binding to RHA, TonEBP and Yc1 were labelled with [³⁵S]methionine using TNT® (transcription/translation)-coupled reticulocyte lysate system kit (Promega). The labelled TonEBP or Yc1 was incubated with GST-RHA fragment-agarose in lysis buffer for 1 h at 4°C with gentle mixing. The mixtures were then washed with lysis buffer, resolved on an SDS/4–15% gradient polyacrylamide gel and transferred on to a nitrocellulose membrane. The membrane was stained with Ponceau S to visualize protein, and autoradiography was performed in a phosphorimager (Bio-Rad) to visualize [³⁵S]TonEBP or [³⁵S]Yc1 pulled-down.

Transfection

Cells were transfected with various expression plasmids (see above) using Lipofectamine™ 2000 (Life Technologies). To assay TonE-driven transcription, cells grown in each well of a 24-well cluster were transfected with 30 ng of TonE-driven *Photinus* luciferase expression construct (see above), 0.3 µg or 3.0 µg of various combinations of pcDNA-RHA [RHA cDNA in pcDNA3.1 expression vector (Invitrogen)] and empty vector (pcDNA3.1 without insert), and 10 ng of pRL-CMV (Promega) directing *Renilla* luciferase expression driven by the cytomegalovirus promoter. After 4 or 20 h, some of the wells were switched to hypertonic medium. Activity of luciferase from cell lysates was measured using a commercial kit, the Dual-Luciferase® Reporter Assay system (Promega). For each extract, activity of the *Photinus* luciferase was divided by the activity of the *Renilla* luciferase to correct for transfection efficiency.

Immunoblotting and immunoprecipitation

TonEBP antibody was raised against amino acids 78–548 of TonEBP [1]. Anti-RHA antibody [15] was a gift from Dr Jeffrey Parvin. Anti-FLAG and anti-Hsc70 (heat-shock cognate 70 kDa) antibodies were purchased from Sigma-Aldrich. For immunoblot analysis, a nitrocellulose membrane blotted with protein was blocked with blocking solution containing 5% (w/v) non-fat dried milk in T-TBS (Tris-buffered saline with Tween 20: 25 mM Tris/HCl, pH 7.4, 137 mM NaCl, 3 mM KCl and 0.05% Tween 20) for 30 min at room temperature (25°C). The blocked membrane was incubated with primary antibodies dissolved in blocking solution for 1 h. The membrane was washed for 10 min with T-TBS and incubated with the secondary antibody conjugated to alkaline phosphatase (dilution 1:2500) for 1 h. A colorimetric assay was performed to visualize binding of the primary antibody using a commercial kit (Sigma-Aldrich). To detect protein–protein interaction *in vivo*, immunoprecipitation was performed. Cells were washed with PBS or PBS containing an extra 100 mM NaCl for cells cultured in hypertonic medium. Cell lysate was prepared as described above and incubated with appropriate antibody or pre-immune serum for 1 h at 4°C. After addition of Protein A-agarose (Sigma-Aldrich), the mixture was incubated an additional 1 hour. The Protein A-agarose was washed by centrifugation at 10000 g for 10 min, and resuspended in SDS sample buffer for immunoblot analysis.

Immunocytochemistry

mIMCD3 or COS7 cells were grown on glass coverslips for 1 day, before being switched to hypertonic medium for 8 h or

remaining in isotonic medium. The cells were fixed for 15 min in 3% (w/v) paraformaldehyde (Eastman Kodak Company) in PBS and permeabilized for 5 min in 0.5% (v/v) Triton X-100 in PBS. Non-specific binding was blocked with a 20 min incubation in 3% (v/v) FBS in PBS, followed by 1 h of incubation with a 1:2000 dilution of anti-TonEBP antibody or 1:2500 dilution of anti-RHA antibody. Binding of the primary antibody was visualized after a 45 min incubation with a 1:1000 dilution of Alexa Fluor® 568-conjugated goat anti-rabbit IgG (Molecular Probes). The coverslips were washed, mounted using ProLong Antifade kit (Molecular Probes) and observed on a fluorescence microscope.

RNA interference

For RNA interference, 21-nt single-strand RNAs (see Figure 7B) were chemically synthesized and purified using a commercial source (Ambion). To produce siRNA (small interfering RNA) duplexes, 25 μ M single-strand RNAs were incubated in annealing buffer (Ambion) for 1 min at 90 °C followed by 1 h of incubation at 37 °C. HeLa cells were transfected with siRNA as described in [7]. Briefly, HeLa cells in passages 5–10 were grown to 50–70% confluence in six-well plates. Transfection of siRNA was carried out using Oligofectamine™ (Life Technologies) with 200 nM siRNA duplex in the culture medium. Cells were transfected twice with a 16 h interval. After 1 day, 1 μ g of TonE-driven *Photinus* luciferase expression construct and 0.1 μ g of pRL-CMV were transfected in each well using Lipofectamine™ 2000. The next day, some cells were switched to hypertonic medium for 4 h, and immunoblot and luciferase assays were performed.

RESULTS

RHA binds TonEBP

Because TonEBP appeared to be in a large molecular complex [1], we searched for proteins that interacted with TonEBP. We used a GST-fusion of the N-terminal 548 amino acids of TonEBP (Yc1) that included the DNA-binding domain (Rel-homology domain). GST–Yc1 was incubated with cell lysate prepared from Jurkat cells and was pulled-down. Coomassie Blue staining of SDS/polyacrylamide gels revealed that several bands specifically associated with Yc1 (Figure 1A, upper panel). These bands were cut out and analysed by MALDI–TOF–MS. A total of 18 peptides were detected from the band at the 140 kDa position, nine of which matched RHA with coverage of 9% (Figure 1A, lower panel). RHA is a 3'–5' double-stranded DNA/RNA helicase also known as nuclear DNA helicase II [19]. Since RHA is a component of transcription factor complexes including MEF1 (murine elongation factor-1) [20], NF- κ B [21] or mineralocorticoid receptor [22], we decided to examine further the interaction of RHA with TonEBP.

In order to confirm the interaction between Yc1 and RHA, we performed the GST pull-down assay on a smaller scale and examined RHA using a specific anti-RHA antibody. GST–Yc1 consistently pulled-down immunoreactivity of RHA (Figure 1B). The association of Yc1 with RHA was not dependent on the presence of double-stranded DNA or RNA because the addition of ethidium bromide did not affect this binding (Figure 1B, rightmost lane). Ethidium bromide is an intercalating agent that creates distortions in the DNA or RNA double helix interfering with protein binding to DNA [23] or RNA [24]. While GST–Yc1 also pulled-down Ku70 and Ku80, these interactions disappeared in the presence of ethidium bromide (results not shown), indicating that GST–Yc1 interacted with Ku70 and Ku80 via double-stranded DNA. Similar results were obtained when the cell lysate was

treated with DNase I before the pull-down assay: the TonEBP–RHA binding remained while the TonEBP–Ku70/Ku80 binding disappeared (results not shown).

Next, we examined the binding *in vivo* using co-immunoprecipitation assays. First, TonEBP was immunoprecipitated from COS7 cells. The immunoprecipitate contained RHA, indicating that the binding occurred *in vivo* (Figure 1C). We attempted a reverse experiment to examine the pull-down of TonEBP using the anti-RHA antibody. Unfortunately, the anti-RHA antibody was not suitable for immunoprecipitation in that it brought down numerous non-specific proteins. To overcome this, we co-expressed FLAG-tagged RHA (FLAG–RHA) and Myc–TonEBP in HEK-293T cells. When FLAG–RHA was immunoprecipitated by anti-FLAG antibody, TonEBP was also brought down (Figure 1D, rightmost lane). When FLAG–RHA was expressed alone, TonEBP was not detected in the immunoprecipitate (Figure 1D, third lane from the left), most likely because of the low abundance of endogenous TonEBP in HEK-293T cells. These data demonstrate that RHA and TonEBP can pull-down each other in immunoprecipitation assays, indicating that RHA and TonEBP are associated in the cell.

The E'F loop of TonEBP is required for RHA binding

In order to map the site of RHA binding, the c-form of TonEBP [25] was cut into four overlapping fragments as shown in Figure 2(A). Each of the TonEBP fragments, Yc1–Yc4, was fused with FLAG sequence and expressed in COS7 cells. All TonEBP fragments used in the present study (see also below) were characterized previously [17,18]. When immunoprecipitated by anti-FLAG antibody, only Yc1 was associated with RHA (Figure 2B), indicating that RHA bound to the N-terminal 584 amino acids. Serial deletion of Yc1 from the C-terminus (Figures 2D, 2E and 2F) demonstrated that the N-terminal 410 amino acids of TonEBP that lacked the dimerization domain (Figure 2C) bound RHA (Figure 2E). These data indicate that dimerization of TonEBP is dispensable for binding to RHA. On the other hand, deletion upstream of amino acid 410 resulted in the loss of RHA binding, indicating that the transactivation domain at the N-terminus [5] was not involved in the binding. Instead, the data indicate that the E'F loop located at amino acids 389–410 [3] is required for binding to RHA. To pinpoint the binding site, we made several site-directed mutants of Yc1 in which amino acids in the E'F loop were mutated. The mutations were limited to the C-terminal half of the loop because the N-terminal half is involved in the formation of the secondary dimer interface [3]. However, these mutants bound RHA similarly to wild-type Yc1 (results not shown). This is not surprising because the C-terminus of the E'F loop is disordered [3], and the binding might cause it to be ordered. We conclude that the E'F loop of TonEBP is required for binding to RHA, while the domains for dimerization and transactivation are not.

The N- and C-termini of RHA bind TonEBP

In order to map the site of TonEBP binding, fragments of RHA were produced as GST-fusion proteins (Figure 3A). Binding of these RHA fragments to ³⁵S-labelled Yc1 was assayed using GST pull-down analysis. As shown in Figure 3(B), binding was detected in the N-terminal 250 amino acids and in the C-terminal fragment of amino acids 1062–1279. The middle region including the helicase domain did not bind Yc1. Similar results were obtained with ³⁵S-labelled full-length TonEBP (results not shown), indicating that the results shown in Figure 3(B) reflect binding of full-length TonEBP. The N-terminal 250 amino acids are a basic region containing two double-stranded RNA-binding domains [26]. The C-terminal fragment with amino acids 1062–1279

Figure 1 TonEBP interacts with RHA *in vitro* and *in vivo*

(A) Upper panel: GST was fused to the N-terminal 548 amino acids of TonEBP (Yc1) to produce GST–Yc1. GST–Yc1 immobilized on to GSH–agarose was incubated with (+) or without (–) Jurkat cell lysate as indicated. GST–Yc1 was pulled-down and washed by centrifugation, before being subjected to SDS/PAGE and Coomassie Blue staining as described in the Experimental section. Size markers in kDa are shown on the left of the gel. The arrow denotes RHA. Lower panel: the amino acid sequence of human RHA (GenBank® accession number A47363) with peptides detected by MALD–TOF underlined and in bold. **(B)** GST or GST–Yc1 was incubated with the Jurkat cell lysate. Where indicated, 0.1 mg/ml ethidium bromide (EtBr) was added to the incubation mixture. GST was pulled-down, run on an SDS/polyacrylamide gel, and transferred on to a nitrocellulose membrane for Ponceau S staining and immunoblotting (IB) for RHA (α-RHA). **(C)** COS7 cell lysate was immunoprecipitated (IP) using anti-TonEBP antibody (α-TonEBP) or pre-immune serum (PIS). Immunoprecipitates and cell lysate were immunoblotted (IB) for TonEBP and RHA (α-RHA). **(D)** HEK-293T cells were co-transfected with FLAG-fused RHA and Myc-fused TonEBP. Cell lysate was immunoprecipitated (IP) using anti-FLAG (α-FLAG) antibody and then immunoblotted (IB) for FLAG and TonEBP (α-TonEBP).

contains the glycine-rich region capable of binding single-strand nucleic acids [19]. This region also contains sequences for nuclear localization and nuclear export [27].

Hypertonicity reduces RHA–TonEBP binding

We investigated whether RHA binding to TonEBP was affected by hypertonicity. RHA binding to TonEBP was assayed using co-immunoprecipitation assays, as in Figure 1(B), at various times after COS7 cells were switched to hypertonic medium. In four independent experiments, exposure to high salt failed to increase the amount of RHA recovered by immunoprecipitation of TonEBP (Figure 4), while the abundance of TonEBP increased after 4 h as reported previously [6]. At 8 h, when the abundance of TonEBP doubled, the amount of RHA that was co-immunoprecipitated with TonEBP remained the same, as indicated by the decrease in the RHA/TonEBP ratio (Figure 4, lower panel). These data

suggest the possibility that the dissociation of RHA is involved in the activation of TonEBP in hypertonicity.

Does DNA binding affect the RHA–TonEBP binding?

Since RHA appeared to bind the E/F loop of TonEBP that formed a secondary dimer interface that is involved in complete encirclement of DNA [3], we asked whether DNA binding would affect the interaction with RHA. To answer this question, we attempted to saturate DNA binding of TonEBP by incubating COS7 cell lysates with a DNA of 23 bp containing a TonE sequence (CTTGG-TGGAAAATTACCGCTGGT; TonE is underlined) at 500 nM which exceeded its *K_m* more than 10-fold [18]. As a control, a DNA containing a mutant TonE sequence (CTTGGTtGAAAATTACCGCTGGT; mutated residue in lower case) which displayed negligible affinity to TonEBP [18] was used. In several independent experiments, the wild-type TonE or mutant TonE

Figure 2 Mapping the RHA interaction site on TonEBP

(A) Schematic representations of TonEBP (top) and its fragments. In Yc2, Yc3 or Yc4, the N-terminal 76 amino acids were fused to the fragment indicated by the amino acid numbers on the right. RHD, Rel-homology domain; Q, glutamine repeat. (B) COS7 cells were transfected with FLAG-fused constructs as indicated. Cell lysates were immunoprecipitated (IP) with anti-FLAG (α -FLAG) antibody and immunoblotted (IB) for RHA (α -RHA) and FLAG. (C) Detailed structure of Yc1. NLS, nuclear localization signal; arrow, E'F loop at amino acids 389–410. (D, E and F) The indicated FLAG fusions were transfected into COS7 cells and analysed as in (B).

did not consistently affect the binding to RHA (Figure 5). These data do not support the model in which DNA binding affects the association between TonEBP and RHA.

RHA is constitutively nuclear

The C-terminus of RHA, one of the sites where TonEBP binds (see above), regulates nuclear import and export for RHA [27]. This region is also critical for the role of RHA in the bidirectional nucleocytoplasmic transfer of a retroviral transcript [28]. We asked whether RHA played a role in the nuclear translocation of TonEBP in response to hypertonicity. To answer the question, we examined the distribution of RHA in the nucleus and cytoplasm. Immunoblot analysis of the nuclear and cytoplasmic fractions prepared from COS7 cells revealed that RHA was predominantly localized in the nucleus both in isotonic and hypertonic conditions (Figures 6A and 6B). This observation was not due to an artifact of experimental procedures, because, in the same preparations, TonEBP moved from cytoplasm to the nucleus as reported previously [29]. Furthermore, the observations shown in Figures 6(A) and 6(B) were confirmed by immunohistochemical analysis of COS7 cells (results not shown) and mIMCD3 cells (Figure 6D). Thus RHA is constitutively nuclear, while TonEBP distribution varies depending on ambient tonicity. We next asked whether TonEBP in the cytoplasm was bound to RHA.

Immunoprecipitation of the cytoplasmic and nuclear fractions revealed that TonEBP was associated with RHA both in the cytoplasm and the nucleus with equal efficiency (Figure 6C). In summary, TonEBP changes nucleocytoplasmic distribution depending on the ambient tonicity, while RHA remains nuclear. These data do not support the model in which RHA plays a role in the nucleocytoplasmic trafficking of TonEBP.

Overexpression of RHA inhibits TonEBP in hypertonicity

To investigate the functional consequence of RHA binding to TonEBP, we examined the effects of overexpression of RHA on TonEBP activity measured by expression of a luciferase reporter gene driven by TonE (TonE–luciferase reporter) [18]. In various cultured cells, including mIMCD3, HeLa, COS7 and HEK-293T cells, co-transfection of RHA along with the TonE–luciferase reporter resulted in a reduced expression of luciferase expression up to 90% compared with co-transfection of expression vector without RHA (results for HEK-293T cells are shown in Figures 7A and 8). This inhibition was dose-dependent and was evident in hypertonic conditions. These data demonstrate that overexpression of RHA inhibits the activity of TonEBP in hypertonicity.

To explore further the role of RHA, we knocked down expression of RHA using siRNA targeted to nucleotide 255 of RHA

Figure 3 Mapping TonEBP-interaction sites on RHA

(A) Schematic representations of human RHA (top) and its fragments (amino acid numbers are shown on the right) used for GST fusion. dsRBD, double-stranded RNA-binding domain; helicase, catalytic domain; RGG, region rich in glycine. (B) Pull-down of Yc1 by various GST-fusion proteins. Yc1 was labelled with [³⁵S]methionine by *in vitro* translation and incubated with various GST-fusion proteins as indicated. GST was pulled-down, run on an SDS/polyacrylamide gel and transferred on to a nitrocellulose membrane. The upper panel is an autoradiogram of the membrane showing [³⁵S]Yc1 (arrow). The lower panel is a Ponceau S staining of the membrane. In the leftmost lane, marked 'input', 20% of [³⁵S]Yc1 used for each pull-down reaction was loaded.

Figure 4 Effects of hypertonicity on the interaction between TonEBP and RHA

COS7 cells were switched to hypertonic medium made by the addition of 100 mM NaCl for 1 (H1), 4 (H4) and 8 h (H8) or remained in control isotonic medium (I). The cell lysate was immunoprecipitated (IP) using anti-TonEBP antibody or pre-immune serum (PIS). The immunoprecipitates were immunoblotted (IB) with antibodies against TonEBP (α-TonEBP) and RHA (α-RHA) as indicated in the top panels. The signal in the immunoblot was quantified using QuantOne software (Bio-Rad), and relative ratios of RHA/TonEBP are shown in the bottom panel.

Figure 5 Effects of DNA binding on the interaction between TonEBP and RHA

Cell lysate was prepared from COS7 cells cultured in isotonic (Iso) or hypertonic medium (Hyper) for 18 h. Where indicated, the lysate was incubated for 3 h at room temperature with 500 nM of double-stranded oligonucleotide containing wild-type (WT) or mutant (Mut) (incapable of binding to TonEBP) TonE. The lysates were then immunoprecipitated (IP) with anti-TonEBP antibody (α-TonEBP) or pre-immune serum (PIS), and the precipitates were immunoblotted for TonEBP and RHA (α-RHA).

mRNA (Figure 7B). Another siRNA (SCR) that did not target TonEBP was used as a negative control. The 255 siRNA knocked down expression of RHA (Figure 7C, middle panel). The knock down was more effective in hypertonic conditions compared with isotonic conditions. As expected, SCR siRNA did not affect expression of RHA. These effects were specific in that expression of Hsc70 was not affected by either siRNA (Figure 7C, bottom panel). Expression of the TonE-driven luciferase reporter was not affected in cells transfected with either the 255 or SCR siRNA (Figure 7D). We conclude that a reduction in the level of RHA expression by itself does not affect the activity of TonEBP.

Catalytic activity of RHA is not required for inhibition of TonEBP

We examined the K417E mutant RHA that is defective in nucleotide binding [16] to investigate whether catalytic activity is required for the inhibition of TonEBP. This mutant was as effective as the wild-type RHA in inhibiting the activity of TonEBP at comparable levels of expression (Figure 8). When we examined effects of the RHA fragments shown in Figure 3(A), we found that only the N-terminal fragments (residues 1–250 and 425–663) inhibited TonEBP moderately, and much less effectively than the full-length RHA (results not shown). Thus full-length RHA is required for the inhibition of TonEBP, but the catalytic activity is dispensable.

DISCUSSION

Collectively, the data demonstrate that RHA binds to TonEBP. The N- and C-termini of RHA appear to bind the E'F loop of TonEBP. While TonEBP constitutively forms a dimer via the C-terminus of RHD, dimerization is not required for the binding to RHA. RHA does not interact with any of the transactivation domains of TonEBP. Overexpression of RHA inhibits TonEBP activity, while knockdown of RHA does not affect it. Catalytic activity of RHA is dispensable for the inhibition of TonEBP. We speculate that overexpression of RHA leads to increased binding to TonEBP in hypertonicity, counteracting the decrease in binding (Figure 4). This can explain the dose–response inhibition of TonEBP in hypertonicity. The lack of effects by knockdown of RHA can be explained as follows: because the transient knockdown is

Figure 6 RHA is constitutively nuclear

(A, B) Cytoplasmic (C) and nuclear (N) extracts were prepared from COS7 cells cultured in isotonic (Iso) or hypertonic medium (Hyper) for 18 h and immunoblotted (IB) for TonEBP (α -TonEBP) and RHA (α -RHA). Band intensity was quantified as described above. (C) TonEBP was immunoprecipitated (IP) from the samples shown in (A), except that nuclear (N) and cytoplasmic (C) extracts from isotonic (Iso) medium were increased 10- and 2-fold respectively. The precipitates were immunoblotted for TonEBP (α -TonEBP) and RHA (α -RHA). The leftmost lane, labelled 'PIS', is the nuclear extract from hypertonic (Hyper) cells immunoprecipitated with pre-immune serum. (D) mIMCD3 cells were grown on glass coverslips and cultured in isotonic (Iso) or hypertonic (Hyper) medium for 8 h. The cells were fixed, permeabilized and immunostained for RHA and TonEBP.

due to a reduced synthesis, RHA already bound to TonEBP is unaffected. We suggest that the decrease in RHA–TonEBP binding in response to hypertonicity is another pathway to the stimulation of TonEBP in addition to nuclear translocation and transactivation.

The E/F loop of TonEBP is a region of 22 amino acids that connect the E' and F β -sheets of the Rel-homology domain. The E/F loops of TonEBP form a secondary dimer interface that

Figure 7 Effects of overexpression and knockdown of RHA on TonEBP activity

(A) HEK-293T cells were co-transfected with TonE-driven luciferase reporter along with increasing amount of RHA expression plasmid (RHA) or empty vector (Vector). Empty vector was added to equalize the total amount of DNA in all conditions. After overnight culture, the cells were treated with hypertonic medium made by the addition of 100 mM NaCl for 4 h (Hyper) or remained in control isotonic medium (Iso). The relative activity of luciferase corrected for transfection efficiency is shown as means $\pm$ S.D. ($n = 3$). * $P < 0.05$ compared with 'Vector'; # $P < 0.01$. (B) Structure of siRNA duplexes targeted to human RHA. '255' is targeted to nucleotides 255–273 of RHA cDNA (GenBank® accession number L13848) or amino acids 58–65. 'SCR' is a random sequence for a negative control. (C, D) HeLa cells were transfected twice with a 16 h interval with 200 nM siRNA or buffer as indicated. After 24 h, the cells were transfected with TonE–luciferase reporter. The next day, half of the cells were switched to hypertonic medium for 4 h (Hyper) and the rest remained in control isotonic medium (Iso). (C) Some cells were immunoblotted (IB) for TonEBP (α -TonEBP), RHA (α -RHA) and Hsc70 (α -Hsc70) and (D) others were assayed for luciferase as in (A).

encircles the DNA. Because of this encirclement, dissociation of DNA is >10 times slower in TonEBP than in NFAT1 or NF- κ B [3]. The overall conformation of the E/F loop in TonEBP is similar to that of NFAT1 [3]. Interestingly, the E/F loop of NFAT1

Figure 8 Effects of K417E RHA on TonEBP activity

HEK-293T cells were co-transfected with TonE-luciferase reporter along with varying amounts of empty vector alone (pcDNA3.1) or vector containing FLAG-tagged wild-type (RHA-WT) or mutant (RHA-K417E) RHA. An immunoblot of FLAG is shown beneath the histogram which shows the activity of luciferase under isotonic (Iso) and hypertonic (Hyper) conditions. See Figure 7 for other details.

mediates the interaction with AP1 (a Fos–Jun heterodimer) to form the NFAT1–AP1 complex [30]. The key amino acid residues involved in the interaction with AP1 are not conserved in the E/F loop of TonEBP, suggesting that TonEBP does not bind AP1. Although quite different from the E/F loops of TonEBP and NFAT, the region comparable with the E/F loop of NF- κ B interacts with the high-mobility group protein HMG-I(Y) [31]. This interaction stabilizes the NF- κ B–DNA interaction and stimulates transcription. In this regard, it is not surprising that the E/F loop of TonEBP interacts with RHA. While DNA binding enhances the NFAT1–AP1 or NF- κ B–HMG-I(Y) interaction, the data in Figures 1(B) and 5 do not support such a model for the TonEBP–RHA interaction.

How RHA inhibits the activity of TonEBP is of great interest. RHA unwinds double-stranded DNA and double-stranded RNA in the 3'–5' direction *in vitro* [19]. In the nucleus, RHA is constitutively associated with RNA [32] and is involved in various aspects of RNA processing, such as RNA splicing and RNA editing [19]. In evolution, RHA has diverged from other DEXH (Asp–Glu–Xaa–His) proteins by acquiring two additional nucleic-acid-binding domains: two double-stranded RNA-binding domains at the N-terminus and the RGG (Arg–Gly–Gly) domain at the C-terminus [33]. RHA has also been implicated in transcriptional stimulation. The *maleless* protein of *Drosophila*, the dipteran orthologue of RHA, stimulates expression of the genes in the single X chromosome in males [34]. In mammalian cells, RHA bridges CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 [15] and BRCA1 [35] to RNA polymerase II. RHA is a component of the transcription factor complex containing NF- κ B [21], MEF1 [20] or mineralocorticoid receptor [22]. RHA specifically binds the transactivation domains of NF- κ B [21] and mineralocorticoid receptor [22]. As such, overexpression of RHA stimulates transcription mediated by CREB, NF- κ B, MEF1 and mineralocorticoid receptor. Catalytic activity is required for this effect in that a K417R mutant, similar to the one used in Figure 8, inhibits the activity of NF- κ B [21]. The action of RHA on TonEBP is quite different from its action on other transcription factors described above. To our knowledge, TonEBP is the first transcriptional mechanism inhibited by overexpression of RHA. This is consistent with the lack of interaction between RHA and transactivation domains of TonEBP. Since this inhibition does not require its catalytic activity,

we suspect that interaction of RHA with other proteins is involved in the inhibition. For example, RHA binds topoisomerase II α in a manner independent of the helicase activity [16]. This might be relevant because supercoiling of DNA is implicated in the stimulation of gene transcription in response to hypertonicity in bacteria [36]. Understanding how RHA inhibits TonEBP is likely to provide novel insight into the hypertonicity signalling to TonEBP.

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